3491-Pos
Imaging the Disassembly of Syntxin Cluster during the Exocytosis of Single Secretory Granules
Mitsuharu Midorikawa, Wolfhard Almers.
Oregon Health & Science University, Portland, OR, USA.
During the exocytosis of synaptic vesicles or of secretory granules, three so-called SNARE proteins mediate membrane fusion. One of the SNARE proteins, Syntaxin (Syx) is known to reside in the plasma membrane. Our previous work has shown that Syx makes a submicron size cluster where a granule docks at the plasma membrane. By labeling Syx clusters and granules in different colors, we now show that the Syx cluster disassembles when the adjacent granule undergoes exocytosis.
As a model system, we used Ins-1 cells, a cell line derived from pancreatic beta cells. Cells expressed tissue plasminogen activator-EGFP (tPA-EGFP) to mark granules in green, and Syx-mCherry to mark Syx in red. Cells were voltage clamped in the whole-cell mode and exocytisis was evoked by depolarizing pulse. Exocytosis of single granules was observed with TIRF. Some granules (''responders'') brightened suddenly, as their exocytosis caused the release of protons and the de-quenching of tPA-EGFP. Other granules remained quiescent and served as controls (non-responders). The fluorescence of Syx-mCherry was measured at the same times and locations as that of the granules. Before exocytosis, the Syx-mCherry signal was significantly brighter beneath responders than beneath non-responders. This is consistent with the idea that syntaxin nanodomains facilitate exocytosis. After exocytosis, the Syx-mCherry signal beneath responders diminished, while no large change occurred for non-responders. Some ''responders'' were not visible before fusion, suggesting that they were located apart from the plasma membrane. Even for such granules, Syx-mCherry signal was observed before exocytosis and diminished after exocytosis. These results indicate that Syx clusters beneath granules are an advantage for exocytosis but then disassembled at the time of granule fusion.

3492-Pos
Single Molecule Study of Disassembly of SNARE Complex by NSF/α−SNAP
Jaemyeong Jung[1,2], Timothy D. Fenn[1], Steven Chu[3,4], Axel T. Brunger[1,2].
[1]Departments of Molecular and Cellular Physiology, Neurology and Neurological, Structural Biology, and Photon Science, Stanford University, Stanford, CA, USA, [2]Howard Hughes Medical Institute, Stanford University, Stanford, CA, USA, [3]Lawrence Berkeley National Lab and Departments of Physics and Molecular and Cell Biology, University of California, Berkeley, CA, USA, [4]Present address: Department of Energy, 1000 Independence Avenue SW, Washington, D.C., MD, USA.
In eukaryotic cells, material exchange between distinct organelles within the cytosol is required for many biochemical processes. The phospholipid vesicles containing the materials are transported to the target membrane and merged. All known forms of intracellular membrane fusion or vesicle fusion involve a highly conserved family of proteins termed SNAREs (Soluble N-ethyl maleimide sensitive-factor Attachment Protein Receptors). The assembly and dissociation of SNARE proteins drives membrane fusion. A good example is chemical release in the synapse, which is essential for inter-communication between neurons. Although it has been known that the ATPase activity of NSF (N-ethyl maleimide Sensitive-Factor) is involved in the disassembly of SNARE complex, the molecular details of the reaction are not understood. In order to investigate the molecular mechanism, we applied a fluorescence-based *in vitro* approach at the single molecule level. SNARE complexes from three neuronal proteins (Syntanxin1a, Snap-25, and Synaptobrevin) were labeled with suitably chosen FRET dye pairs based on the available structural information about the SNARE complex. For the particular labeling pairs, high FRET corresponds to assembled complex, low FRET corresponds to dissociated complex. To test the labels, we used a conventional bulk FRET assay and found a progressive temporal change from high FRET to low FRET states, indicating complex dissociation into each individual protein in a Mg^{2+}, ATP, and α-SNAP dependent manner. Using a single molecule TIRF optical setup, the same reactions were monitored, and likewise show a change in FRET signal corresponding to complex disassembly. We are studying the disassembly reaction for a number of different labeling pairs along the SNARE helical bundle. Single molecule kinetic studies are underway to understand the molecular mechanism of NSF catalyzed SNARE protein disassembly.

3493-Pos
A Pre-Docking Role for Microtubules in Insulin-Stimulated Glucose Transporter 4 Translocation
Yu Chen, Yan Wang, **Wei Ji**, Pingyong Xu, Tao Xu.
Institute of Biophysics, Chinese Academy of Science, Beijing, China.
Insulin stimulates glucose uptake by inducing translocation of glucose transporter 4 (GLUT4) from intracellular resides to the plasma membrane. How GLUT4 storage vesicles are translocated from the cellular interior to the plasma membrane remains to be elucidated. In the present study, intracellular transport of GLUT4 storage vesicles and the kinetics of their docking at the plasma membrane were comprehensively investigated at single vesicle level in control and microtubule-disrupted 3T3-L1 adipocytes by time-lapse total internal reflection fluorescence microscopy. It is demonstrated that microtubule disruption substantially inhibited insulin-stimulated GLUT4 translocation. Detailed analysis reveals that microtubule disruption blocked the recruitment of GLUT4 storage vesicles to underneath the plasma membrane and abolished the docking of them at the plasma membrane. These data suggest that transport of GLUT4 storage vesicles to the plasma membrane takes place along microtubules and that this transport is obligatory for insulin-stimulated GLUT4 translocation.

3494-Pos
A Short Lipopeptide Derived from the N-Heptad Repeat Region Inhibits HIV-1 Gp41 Mediated Fusion Via an Altered Mode of Action
Avraham Ashkenazi, Yael Wexler-Cohen, Yechiel Shai.
Weizmann Institute of Science, Rehovot, Israel.
Human immunodeficiency virus (HIV-1) fusion is mediated by the gp41 subunit of the envelope glycoprotein (ENV). Folding into the post-fusion conformation is apparently the rate limiting step for the fusion reaction and it enables inhibition of the fusion process. This is demonstrated by the inhibitory capability of N- or C-peptides derived from the N-or C-terminal heptad repeat (NHR or CHR) regions, respectively. These peptides have been shown to bind their endogenous counterparts thereby preventing progression into the post-fusion conformation and arresting fusion. Since N-peptides tend to aggregate, C-peptides are more potent fusion inhibitors, and therefore, attracted most therapeutic effort and research. Here we show that a short peptide derived from the classical NHR region is highly potent inhibitor only when linked to a fatty acid at the N- and not the C-terminus. To our knowledge, this is the shortest potent inhibitory peptide derived from the NHR region. Using biophysical and cellular approaches, we: (i) revealed that its mode of action is altered from other classical N-peptide, and (ii) suggest a new motif in the NHR involved in stabilization of the post-fusion conformation. Besides shedding light on the mechanism of HIV-cell fusion, the similarity between the ENV of lentiviruses provides a new approach for designing short inhibitors from the NHR region of other viruses as well.

3495-Pos
HIV-1 Env-Mediated Membrane Fusion Monitored by Fluorescence Microscopy of Syncytium Formation Between Clone69T1RevEnv and SupT1 Cells
Michael Yee[1], Krystyna Konopka[1], Jan Balzarini[2], **Nejat Düzgüneş**[1].
[1]Department of Microbiology, University of the Pacific Arthur A. Dugoni School of Dentistry, San Francisco, CA, USA, [2]Rega Institute for Medical Research, Catholic University of Leuven, Leuven, Belgium.
Objectives: HIV-1 initiates infection by fusing with CD4+ lymphocytes or macrophages, mediated by the envelope protein Env (gp120/gp41). We developed a new HIV fusion assay using fluorescently labeled Clone69T1RevEnv cells expressing Env, and CD4+ SupT1 cells. We examined whether known inhibitors of HIV-1 infection, including peptides, lectins and antibodies inhibit membrane fusion in this system.
Methods: Clone69TRevEnv (''HIV-Env'') cells were maintained in DMEM with heat-inactivated fetal bovine serum (FBS), L-glutamine, geneticin, hygromycin B and tetracycline. Env expression was induced by removing tetracycline. Cells were plated in 48-well plates for 24 h and labeled with Calcein-AM Green. SupT1 cells, maintained in RPMI 1640 with 10% FBS, penicillin, streptomycin and L-glutamine, were labeled with CellTrace(tm) Calcein red-orange. The SupT1 cells were incubated with adherent HIV-Env cells, with or without the inhibitors, for 3 h, and observed under a Nikon fluorescence microscope. Syncytia formation resulted in orange fluorescence.
Results: Hippeastrum hybrid (Amaryllis) agglutinin (HHA), Galanthus nivalis (Snowdrop) agglutinin (GNA), and the peptide Enfuvirtide inhibited membrane fusion at 1 μg/ml. Anti-gp120 (IgG1B12, m14 IgG, and 2G12), and anti-gp41 (2F5 and 4E10) antibodies that inhibit HIV-1 infection, had little or no inhibitory effect on cell-cell fusion.
Conclusion: Fluorescently labeled HIV-Env and SupT1 cells can be used to monitor HIV Env-mediated fusion as the fused cells change color. This assay can be adapted to screen novel inhibitors of membrane fusion in high-throughput assays. Our observation that antibodies that inhibit HIV infection do not inhibit syncytium formation, suggests that the mechanisms of cell-cell and virus-cell membrane fusion may be different. This observation also raises the possibility that the antibodies may not be able to inhibit the spread of viral genetic material from infected cells to uninfected cells.